ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Forty-third Quarterly Report of Progress

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Contributors:

A. L. Reyes
A. J. Wehby
R. G. Crawford
J. C. Wimsatt
J. E. Campbell
J. T. Peeler

Report Prepared by:

A. L. Reyes

A. L. Reyes, Microbiologist

Report Submitted and Forwarded By:

J. E. Campbell

J. E. Campbell, Ph.D.
Principal Investigator

Thermal Resistance of Bacillus subtilis var. niger in a Closed System

J. T. PEELER, A. L. REYES, R. G. CRAWFORD,

A. J. WEHBY, and J. E. CAMPBELL

Division of Microbiology
Food and Drug Administration
Cincinnati, Ohio 45226

ABSTRACT

The heat resistance of Bacillus subtilis var. niger has been measured from 85 to 125 C using moisture levels of % RH $\leq$ 0.001 to 100 in a closed system. Five curves have been presented to characterize the thermal destruction, using thermal death times defined as F values at a given combination of three moisture and temperature conditions. Reductions of 99.99% (4 $\log_{10}$ cycles) of the initial population were estimated for the three moisture conditions. At 110 C, the expected time for a four $\log_{10}$ reduction was 1.1 h at % RH = 100, 3.1 h at % RH $\leq$ 0.07, and 54 h at % RH = 10.7. Goodness of fit tests to examine the adequacy of three polynomial models failed to indicate a trend. The linear model (from which estimates of D are obtained) was satisfactory for estimating the thermal death times (% RH $\leq$ 0.07) in the plate count range. The estimates based on observed thermal death times and D values for the % RH = 100 diverged so that D values, generally gave a more conservative estimate over the temperature range 90 to 125 C. Estimates of Z_F and Z_L ranged from 32.1 to 58.3 C for the % RH of $\leq$ 0.07 and 100. A Z_D = 30.0 was obtained for data observed at % RH $\leq$ 0.07. The Z_F results

were obtained from plotting observed log times to achieve a 99.99% reduction in the initial population versus temperature. Estimates of Z_L and Z_D were obtained by using linear estimates of $L_{100} \simeq 4D$ and D values in a similar plot.

Thermal Resistance of *Bacillus subtilis* var. *niger* in a Closed System

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The thermal resistance of microorganisms has been measured since 1920 using two physical parameters, time and temperature. Murrell and Scott (5) introduced water activity (moisture content of a system) as a third physical variable. Other factors, such as pH, have been considered, but they have less influence on the thermal resistance of microorganisms. These three physical parameters have been studied in more detail since the National Aeronautics and Space Administration (NASA) designed an unmanned planetary capsule to land on the Martian surface. This capsule is to be dry-heat sterilized before launch in an inert-gas environment. The *Bacillus subtilis* var. *niger* was one strain chosen to specify the parameters of dry-heat sterilization.

Representative of recent work are reports by Angelotti et al. (2), Paik et al. (7), Wardle et al. (12), and Simko et al. (11), that describe the thermal destruction of *B. subtilis* var. *niger*. Measures of thermal resistance were obtained in plastic capsules, between mated surfaces, and from selected lander capsule surfaces. Thermal resistance was reported in terms of D and Z values as defined in Schmidt (9) and was measured at temperatures between 105 to 160 C.

The present investigation was undertaken in a closed can system over a temperature range of 85 to 125 C, with moisture content defined as percent relative humidity (% RH) at temperature from < 0.001% to 100%. Curves obtained from this range of temperatures and % RH were, in general, nonlinear on a semi-log plot of survivors versus time at constant temperature. The objective of this study was to determine the effect of moisture content in a closed system on thermal resistance of B. subtilis var. niger spores. A secondary objective was to explore ways of expressing the thermal properties of the spores. Empirical studies are reported comparing the thermal resistance in terms of thermal death times at various moisture conditions and temperatures (defined here as an F value), and D values (9).

MATERIALS AND METHODS

Production of spores and description of closed system. The Bacillus subtilis var. niger spores were surface grown on nutrient agar as described by Angelotti et al. (2). The spores were washed from the surface with double-distilled sterile water and the spore suspension was shaken with glass beads, filtered through cotton, and held at 45 C (water bath) overnight. The heated suspension was washed and stored at 5 C in 95% ethyl alcohol.

The closed system consisted of a 2-1/4-inch (5.72 cm) x 2-7/8-inch (7.30 cm) diameter tin can with four stainless steel circular shelves. Thirty cups fit on a single shelf and 120 in the can. This quantity of cups allows the experimenter the flexibility of observing a few replicates at a time interval in the plate count range or of observing multiple positive and negative results for an MPN estimate.

Bacillus subtilis var. niger spores were suspended in 95% ethyl alcohol, diluted in double-distilled water, and dispensed with a repeating dispenser in 0.01 ml amounts into the stainless steel cups. The inoculated cups, cans, lids, and contents were dried in a vacuum oven for 100 min at 45 to 50 C (1.5-inch Hg pressure absolute). An increasing drying efficiency was achieved by purging the oven with dry nitrogen every 10 min for the first 90 min. This was followed by five consecutive purges of nitrogen with a vacuum cycle between each purge.

After drying, the cans, lids, and contents were removed from the oven and cooled to about 30 C in an equilibration hood. An amount of water or desiccant was placed in the can to achieve the desired % RH at the selected temperatures. The cans were then sealed and removed from the equilibration hood.

Determination of heat resistance. Cans prepared to yield a selected % RH at a particular temperature were completely immersed in a silicone bath operating at the desired test temperature (± 0.1 C). Cans were withdrawn at desired time intervals and plunged immediately into ice water to cool for 15 min. After washing and rinsing, the cans were dried with sterile towels and opened. Viable spores were assayed by sonifying the cups in peptone water. The solution was pipetted into pour plates with TGE agar. The plates were incubated for 48 hr at 35 C.

Statistical calculations. Heat penetration studies were performed and correction for come-up and cool-down times were computed as outlined by Anellis et al. (1). Estimates of linear and polynomial regression and assumptions for calculation are presented by Ostle (6). D values were computed by taking the absolute value of inverse slope estimated from a linear regression of $\log_{10}$

count versus time at constant temperature and RH. D is the time interval at a constant temperature to obtain a 90% reduction in organisms. The observed time to reach a concentration of 100 organisms per cup is defined as F_{100} for a given initial concentration. An estimate of time to reach 100 organisms per cup is defined as $L_{100} = (b_0 - 2)/|b_1|$. This estimate of endpoints assumes the linear model. To obtain Z_D, Z_L, and Z_F, the absolute value of the inverse slope from the linear regression of $\log_{10} D$, $\log_{10} L_{100}$, and $\log_{10} F_{100}$ versus temperature is computed at constant RH. These endpoint estimates (F and L) differ from the usual definition of thermal death time (9), which is observed time of extinction of a given population.

RESULTS

The present study is based on results from 45 experiments. Sixteen runs were observed at 125 C for 16 different moisture contents. Similar results were recorded at 113 C for 15 experiments representing 14 moisture conditions measured as % RH. Data representative of thermal destruction at seven representative moisture conditions are plotted in Fig. 1 and 2. These curves are not least squared fits of the data but are drawn through the points to demonstrate the trends. The description of the least squares analyses to determine a model for the data was inconclusive so that curves in Fig. 1 to 4 are the best visual fits of the points. Fourteen other experiments were performed at six temperatures (85, 90, 95, 100, 105, and 120 C). These runs were performed under dry (% RH $\leq$ 0.1) and wet (% RH = 100) conditions. Figure 3 shows the plot of the average counts per cup for the wet experiments. Three replicate cups were placed on the bottom shelf of the can for each time interval.

Data plotted from these experiments show an irregular pattern. There is, however, a tendency for data collected under dry conditions (Fig. 4) to show a rapid 4 $\log_{10}$ reduction from the initial concentration of about 1 x 10^6 and then tail off to the right. The dry conditions at higher temperatures show more pronounced leveling until the line is almost asymptotic to the base line (Fig. 4). Lines plotted from data of experiments where % RH was near 100 generally showed the opposite effect. There is very little (less than 1 log) decrease in initial population for 10 min (125 C) to 50 min (85 C). After this initial period (Fig. 3), the rate of decrease is rapid.

Least squares analysis. It seemed apparent that one mathematical model would not fit all sets of data. Although there are a number of mechanistic models (i.e., the estimation of values that assume a first order reaction between organisms and heating agent), no one model was found to fit results of these experiments. Thus, the family of polynomial equations was used to empirically fit and screen the data. The linear (D values can be estimated from this model), second, and third order equations were employed. An equation for the general model is given below:

$$y = b_0 + b_1 t + b_2 t^2 + b_3 t^3 + \ldots$$

The $y = \log_{10}$ count and t is the time in hours. Values of b_0, b_1, b_2, etc. are estimated by the method of least squares (6). A goodness of fit test (6) was performed for each set of data and the three models. The goodness of fit test explores the relation between deviations of data from a given model compared to the pooled variance of replicate points. A significant ratio

of these variance estimates means the model does not fit.

The purpose of the empirical curve fits and goodness of fit tests was to screen the data and determine if certain regions of the relative humidity range could be fit by a given order of equation. If a trend were detected, then mechanistic models (one where the coefficients in the model have physical interpretation) could be tested. Goodness of fit test results are listed in the right-hand column of Tables 1 and 2. All tests were performed at the $\alpha = 0.01$ level. A figure "1" in this column indicates that the linear model was satisfactory. A figure "2" or "3" is used when a second or third degree polynomial fits the data, and ** appears when all three models were significant for goodness of fit at $\alpha = 0.01$ level. The frequency of the degree of polynomial chosen is tabulated in Table 3 for wet and dry experimental results.

The majority of the data could not be characterized by any of the three polynomial curve fits. It seems unlikely that a mechanistic model with two to four parameters would yield a better fit. Some mechanistic models, including the double exponential (4,10) and logistic function, were examined by nonlinear (3) methods (not reported here) with the same conclusion.

Calculation of thermal process, D and F values. Since the path of thermal destruction could not be consistently predicted, some path-independent estimation techniques were examined. Schmidt (9) gives a definition of thermal death time (defined here as F value). This F value is the time required to inactivate the initial population. It is common practice to design experiments with 10 thermal death time tubes at various time intervals. Each tube in a group is scored for growth or no growth on removal from an oil or water

bath. The thermal death time has been defined in terms of the time after growth was observed in the last group of tubes and the time when succeeding groups of tubes showed no growth. The F value used here has been given a broader meaning so that the time to reduce an initial population to 100 organisms per cup is called F_{100}, and the time to reduce an initial population to 1 organism per cup could be defined as F_1. Since the path of thermal destruction under constant moisture and temperature conditions is irregular, the redefinition of F allows the thermal resistance to be computed on a common basis. The usefulness of the commonly used D values was also examined for these data. The F value discussed here is a measurement of endpoint for a given concentration. No assumptions are made about the mode of approach to the endpoint. However, it is implied that the curve does not turn back up and the concentration remains the same or decreases after the time observed.

An option of the closed can system is that multiple cups (up to 120 per can) can be processed at once. When the expected count is less than 10 per cup, the cups can be scored for growth or no growth. Plate count and MPN measures could be recorded. The results of the MPN region are too few to report. Observed time required to reach a concentration of 100 is shown in Table 1. The estimates at 100 per cup are based on linear interpolation using the two results bracketing 100. An estimate of the time required to reach 100 by estimating D from the linear model is tabulated also in Table 1. The linear estimates of the time required to reach 100 are shown for 113 and 125 C data in Table 2. These estimates are obtained as follows: The $L_{100} = (b_0 - 2)/|b_1|$, where b_0 and b_1 are the intercept and slope from the linear regression of $\log_{10}$ count (y) and time (t) in hours. The L_{100} is approximately

4D, and the initial concentration was about 1×10^6 organisms per cup for all experiments.

The majority of data at the various temperatures is in either the wet or dry moisture region. Tables 4 and 5 were constructed to summarize the estimated times predicted and observed to reach 100 organisms per cup. The results of a linear regression using these values are plotted in Fig. 5. Figure 5 shows the observed and predicted values for % RH $\leq$ 0.07 and 100 when the concentration per cup is 100 over the temperature range of 85 to 125 C. Two points (113 and 125 C) are plotted for the RH, averaging 10.7%.

DISCUSSION

The use of endpoint estimation provides a method for characterizing the thermal resistance of an organism with respect to RH. Estimates of the time to reach a concentration of 100 can be computed so they are not dependent on the path of the curve. When the D is estimated from the linear model (Y = $\log_{10}$ count and t = time in hours), the estimate of endpoint is dependent on the assumption that the thermal death rate is a first order reaction. This value (L_{100}), referred to as from the linear model, is tabulated in Tables 4 and 5. Observed time (F_{100}) is shown also. The results from the regression of $\log_{10}$ (endpoint in hours) versus temperature in C are plotted (Fig. 5) for both the observed and linear estimates. Estimates of Z value and the predicted line are about the same for observed and linear estimates for the dry data but diverged for observations under wet conditions.

Curves for concentration estimates at 100 per cup (Fig. 5) indicate that the % RH = 100 condition is the most effective condition to inactive B. subtilis compared to the % RH $\leq$ 0.07. It did not matter if the estimates were

endpoints or predicted from the linear model, because the results for wet conditions were below the curves observed in the dry environment. Estimates of Z_F ranged from 32.1 to 58.3. Angelotti et al. (2) found Z_D = 32.0 within stainless steel washers. A Z_D = 30.0 (% RH $\leq$ 0.07) was computed from data in these experiments so that the closed system seems to behave like an enclosed system where the organisms are between two surfaces. There is also an agreement with the Z_F and Z_L which were estimated at % RH $\leq$ 0.07. These values were 32.1 and 32.9.

The linear regression plotted in Fig. 5 is based on the data in Tables 4 and 5. Each line corresponds to a column in these tables. There are only two points for % RH = 10.7, as these values were taken from the linear estimates computed at 113 and 125 C. The implications for choosing a process based on the three physical factors (temperature, time of heating, and % RH) can be examined in these figures. The least effective conditions for inactivation at 113 and 125 C occurred at % RH 5 to 30. Observed and predicted times to achieve inactivation seem to peak at 5 to 10% RH (Table 2). A line for two predicted points, 10.4% RH at 113 C and 10.9% RH at 125 C, is plotted to demonstrate the degree of resistance (Fig. 5).

Estimations of an endpoint at 100 organisms per cup were performed to illustrate the flexibility in characterizing a heat process. These calculations were performed also to compare with estimates from the linear model. In Table 2 (113 and 125 C results) the values observed from linear interpolation of the two points bracketing 100 organisms per cup were 2.4% lower than (not including 100% RH) the estimate based on the linear model. However, the results over temperature for 100% RH (Fig. 5) show a marked divergence in these estimates. The curves in Fig. 3 indicate that linear estimation is definitely inappropriate in this region.

The plots of the number of viable spores versus time as a function of RH (Fig. 1 and 2) emphasize the relative thermal resistance. High and low humidity conditions are clearly the best to use in order to achieve thermal inactivation with the minimum input of heat (calories). Figures 3 and 4 show the thermal inactivation of B. subtilis at the extreme moisture conditions of 100% RH and < 0.001% RH. The temperature range is between 85 and 125 C. It is interesting to observe the difference in shape of the curves. The curves generated from dry conditions (% RH < 0.001) appear to be linear on the semilog plot until they reach a concentration near 1 per cup. Three curves (95, 100, and 105 C), where data are available, then show a flattening out of this concentration (Fig. 4).

Data collected from experiments where the RH is 100% show a different pattern when plotted on semilog paper. The concentration stays constant at the initial concentration of 10^6 organisms per cup up to 50 min for 85 C data (Fig. 3), and then decreases rapidly to 1 organism per cup. This lag in thermal inactivation decreases as the temperature increases until at 125 C, the lag is only about 10 min. These differences between moisture conditions may indicate that the mode of thermal destruction varies. However, the variability of the present experiments did not allow a definitive test for these effects.

Although the shapes of the curves are observed to differ, the method of endpoints can be used to obtain process values. The observed time to reduce the initial population (1×10^6 organisms per cup) to 100 organisms per cup was compared to the analogous estimate from D values. Calculation of D assumes that the data are linear on the semilog plot. Thus the predicted times (Fig. 5) are in agreement with those observed for the dry experiments,

which tend toward linearity but deviate from the wet-side estimates. As noted above, an experimenter would want to avoid the relative humidity regions between these two extremes, since the amount of heat required increases. Two points in the 10% region were plotted at 113 and 125 C to demonstrate this point.

The endpoint curves in Fig. 5 can be used to determine the time to heat a unit. If it is known that a maximum of 10^4 organisms will be on a unit, then a process to reduce the concentration 4 logs might be chosen. Since the wet-heat and dry-heat conditions yield the shortest heating times, the experimenter has a choice of RH conditions. As an example, consider a process where a maximum of 10^4 B. subtilis organisms are known to be deposited per unit. Determine the time to reduce this population to 10^2 per unit under dry-heat conditions at 115 C. Figure 5 gives the time for a 4-log drop (10^6 down to 10^2 organisms per cup) for % RH $\leq$ 0.07 as about 2.1, using the line for observed endpoints. The same estimate for wet heat (% RH = 100) would be 0.5 h. If the experimenter had set the conditions for % RH in the 10 to 11% range, the time to reduce the population to 100 per unit would be about 33 h. The 4-log reduction was chosen for these illustrations since it is known that 10^3 to 10^4 spores (8) are observed from fallout on spacecraft.

Another example of the use of Fig. 5 can be shown by taking approximate estimates of F or L at a constant temperature. Thus, an experimenter performing tests at 110 C (Fig. 5) would expect to take about 1.1 h at % RH = 100, 3.1 h at % RH $\leq$ 0.07, and 54 h at % RH $\cong$ 10.7 to reduce an initial population from 10^6 to 10^2 organisms per cup. It seems unlikely from the goodness of fit tests that a single mechanistic model can be supported over a wide range

of conditions. The estimate of D (supported by only 16% of the goodness of fit tests) seems to be an adequate approximation for many endpoint estimates in this study.

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TABLE 1. Estimates of endpoints at concentrations of 100 for experiments at 85, 90, 95, 100, 105, and 120 C

Temperature (C)	% RH at temp.	Predicted time to reach concentration based on linear model (hr) L_{100}[a]	Observed time to reach concentration per cup (hr) F_{100}	Results of goodness of fit test[b]
85	100	5.5	6.0	**[c]
	100	5.5	6.9	**
90	100	1.	1.8	**
	100	1.7	2.0	**
	100	1.8	1.7	**
	< 0.07	13.6	14.0	**
95	< 0.05	9.1	9.9	1
	< 0.05	9.3	9.0	2
100	100	0.7	0.7	**
	80	2.6	2.5	**
	80	1.9	2.0	**
	< 0.042	5.7	5.1	2
105	< 0.035	4.4	3.2	**
120	0.108	2.9	2.8	**

[a]Estimated time to reach a concentration of 100 per cup using coefficients from the linear model $L_{100} = (b_0 - 2)/|b_1|$.

[b]Results from goodness of fit tests: (1) Linear model test could not be found significant at $\alpha = 0.01$; (2) Second degree model test could not be found significant at $\alpha = 0.01$; and (3) Third degree model test could not be found significant at $\alpha = 0.01$.

[c]** = Significant lack of fit at $\alpha = 0.01$.

TABLE 2. Estimates of endpoints at concentration 100 for experiments at 113 and 125 C

Temperature (C)	% RH at temp.	Predicted time[a] to reach concentration based on linear model (hr)	Observed time to reach concentration per cup (hr)	Results of[b] goodness of fit test
113	< 0.001	1.6	1.5	**[c]
	0.078	3.5	3.3	**
	1.04	14.6	14.6	2
	5.22	46.1	--	1
	10.4	40.0	--	**
	15.7	35.1	31.9	2
	20.9	32.9	31.9	**
	26.1	26.1	28.0	**
	31.3	27.4	--	**
	36.5	15.9	17.2	1
	41.8	9.4	9.0	**
	52.2	4.0	3.5	2
	78.3	0.8	--	3
	100.	1.3	--	2
	100.	1.4	--	2
125	< 0.001	0.4	0.3	**
	0.019	1.0	1.0	3
	0.73	3.4	3.4	3
	3.65	11.1	--	1
	7.3	12.8	12.5	1
	10.9	12.6	12.6	2
	14.6	10.4	10.9	2
	18.2	8.3	7.7	**
	21.9	10.2	10.0	**
	25.5	7.5	7.9	1
	29.1	7.6	--	3
	36.5	2.7	2.5	2
	54.7	0.9	--	1
	73.0	0.9	--	3
	91.2	0.5	0.4	3
	100.	0.6	0.4	**

[a]Estimated time to reach a concentration of 100 per cup using coefficients from the linear model $L_{100} = (b_0 - 2)/|b_1|$.

[b]Results from goodness of fit tests: (1) Linear model test could not be found significant at $\alpha = 0.01$; (2) Second degree model test could not be found significant at $\alpha = 0.01$; and (3) Third degree model test could not be found significant at $\alpha = 0.01$.

[c]** = Significant lack of fit at $\alpha = 0.01$.

TABLE 3. Frequency of experiments in four categories

Category	Model	Wet experiments (% RH = 100)	Dry experiments (% RH $\leq$ 0.07
1	1	0	1
2	2	2	2
3	3	1	1
4	**	9	5

** - All three models were significant for goodness of fit at $\alpha = 0.01$.

TABLE 4. Time (h) observed to reduce the initial population to 100 organisms per cup

Temperature (C)	F_{100} Dry (% RH ≤ 0.07)	F_{100} Wet (% RH = 100)
85	--	6.0
	--	6.9
90	14.0	1.8
	--	1.7
	--	2.0
95	9.9	--
	9.0	--
100	5.1	0.7
	--	--
105	3.2	--
	--	--
113	3.3	--
	--	--
125	1.0	0.4
	--	--
Z_F	32.1	36.7

TABLE 5. Time (h) to reduce the initial population to 100 organisms per cup estimated by linear model

Temperature (C)	$L_{100} = (b_0 - 2)/\lvert b_1 \rvert$	
	Dry (% RH ≤ 0.07)	Wet (% RH = 100)
85	--	5.5
	--	5.5
90	13.6	1.6
	--	1.7
95	9.1	1.8
	9.3	--
100	5.7	0.7
	--	--
105	4.4	--
	--	--
113	3.5	1.3
	--	1.4
125	1.0	0.6
	--	--
z_L	32.9	58.3

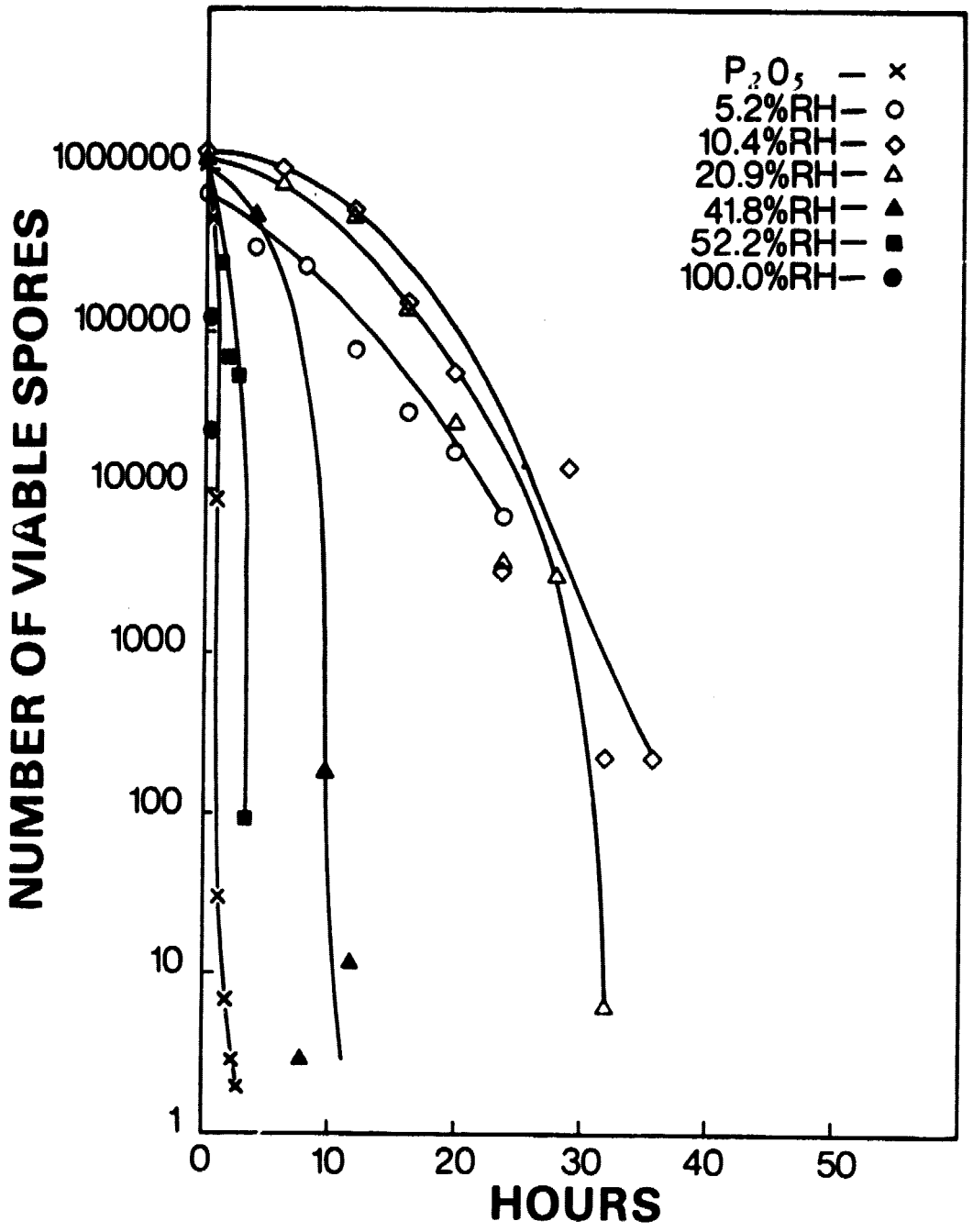

Fig. 1. Thermal inactivation of B. subtilis var. niger in a closed system at seven relative humidities, 113 C.

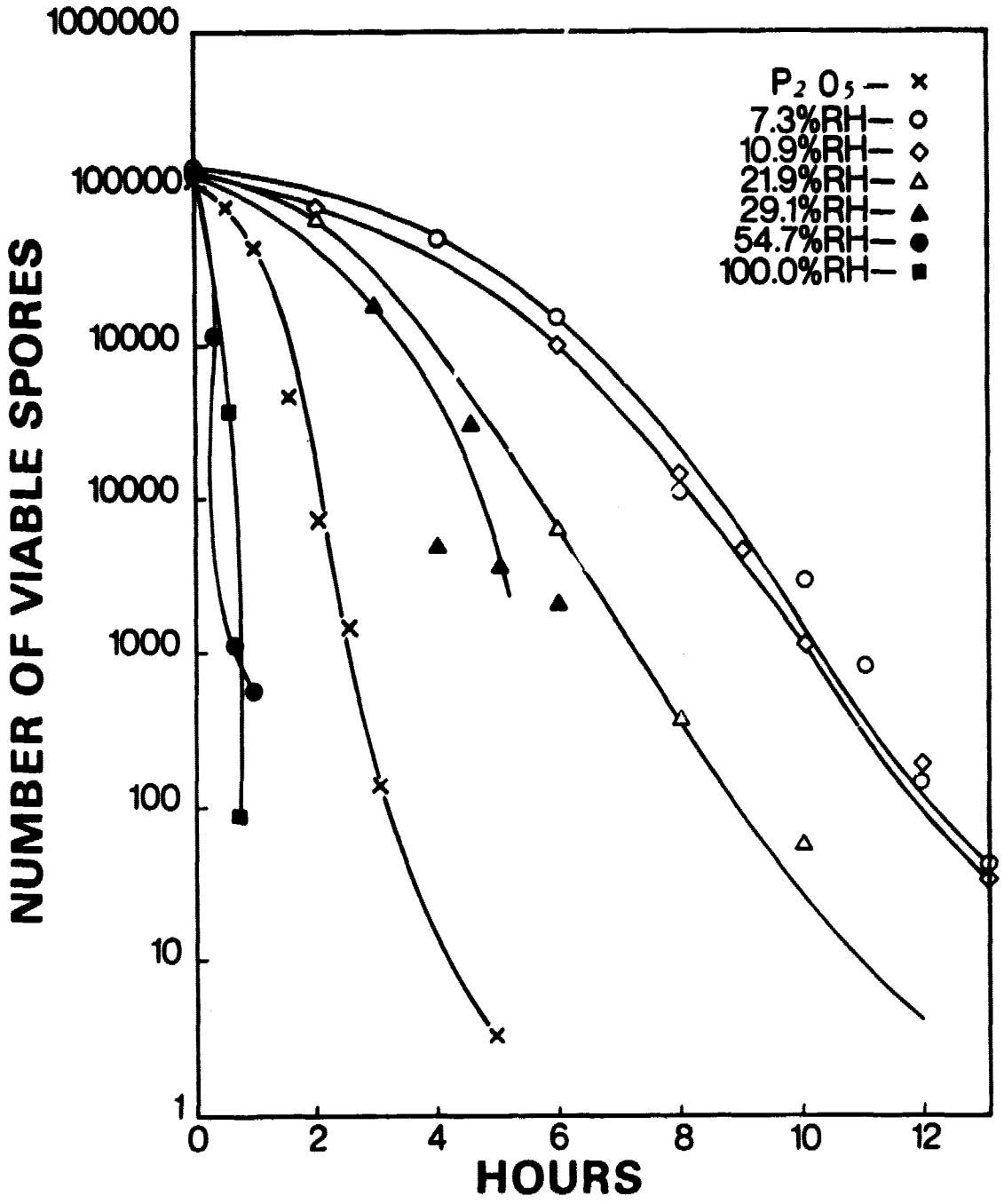

Fig. 2. Thermal inactivation of B. subtilis var. niger in a closed system at seven relative humidities, 125 C.

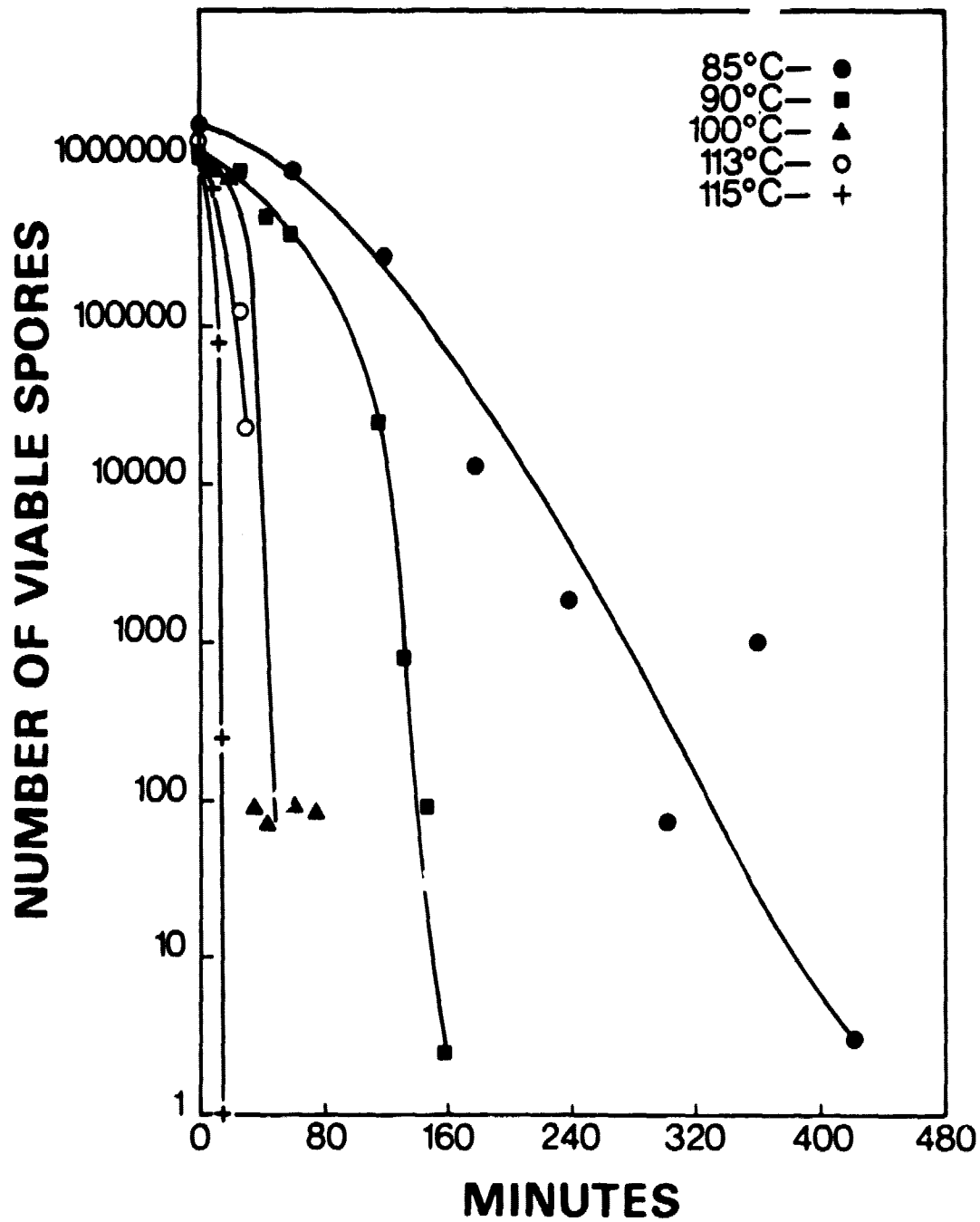

Fig. 3. Thermal inactivation of B. subtilis var. niger in a closed system at five temperatures, 100% RH.

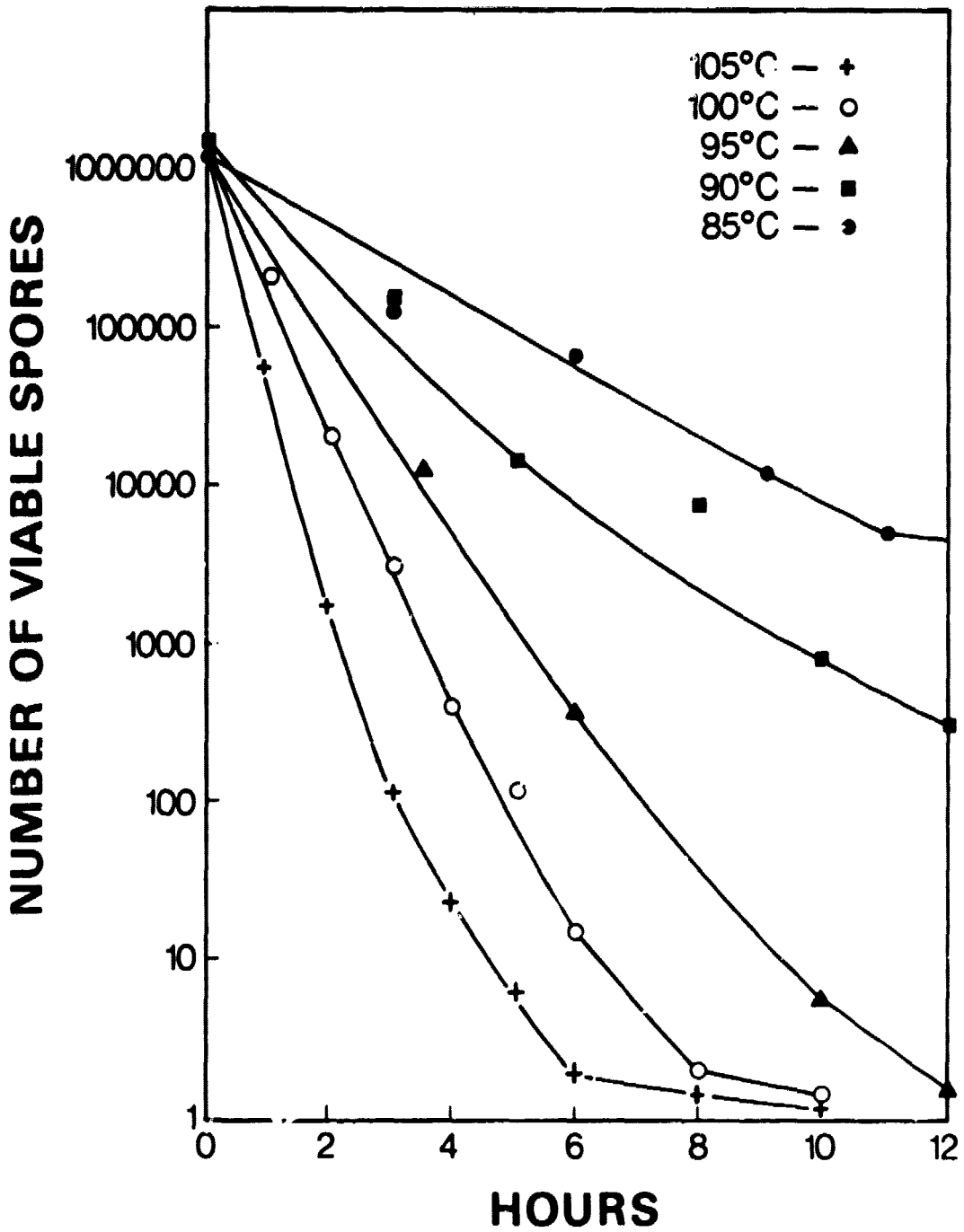

Fig. 4. Thermal inactivation of B. subtilis var. niger in a closed system at five temperatures, < 0.001 % RH.

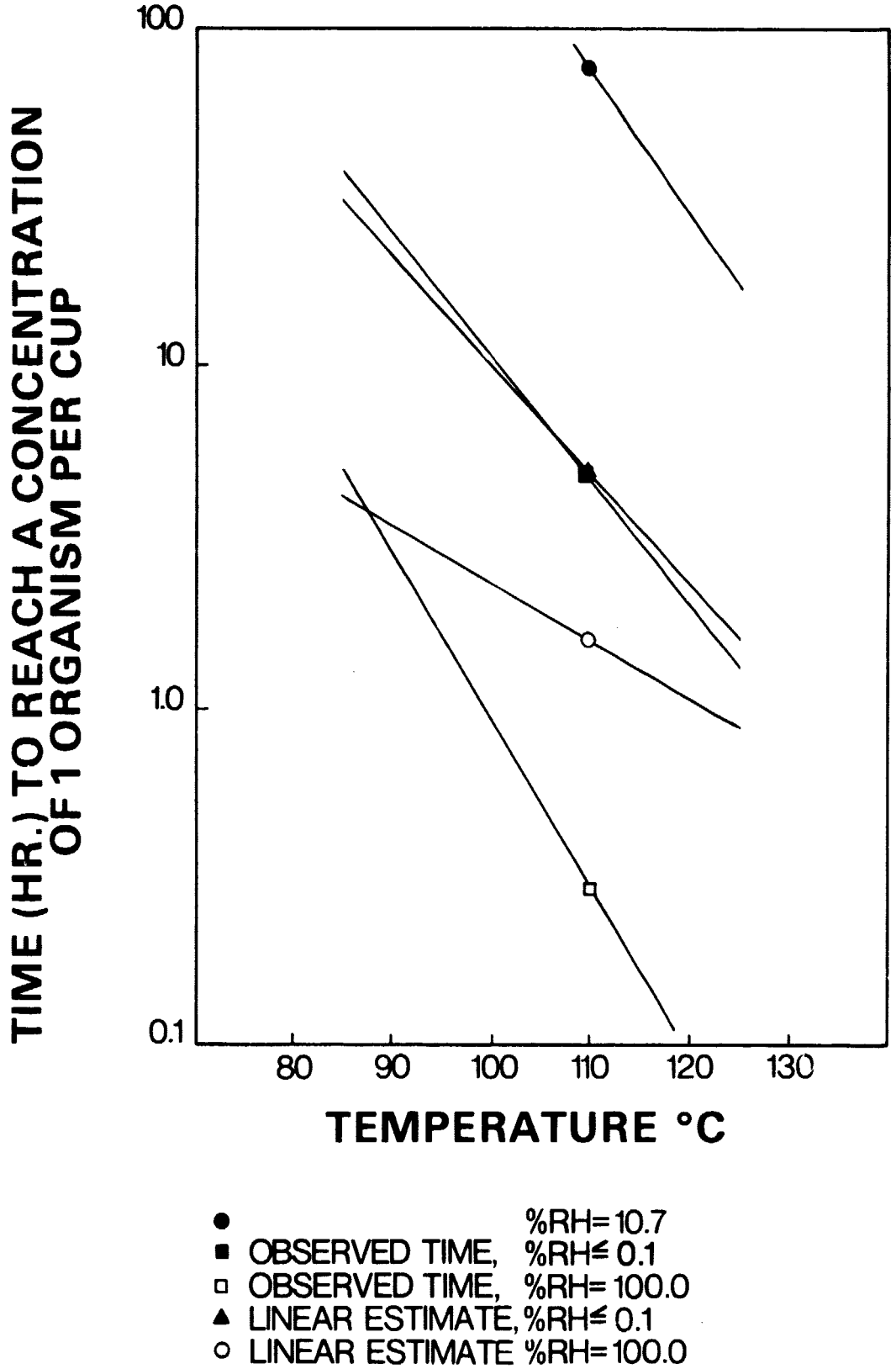

Fig. 5. Relation of the time required to inactivate B. subtilis var. niger from 10^6 to 10^2 organisms per cup versus temperature for three relative humidities. Symbols: ● observed time, % RH = 10.7; ▲ observed time, % RH ≤ 0.11; □ observed time, % RH = 100.0; ■ linear estimate, % RH = ≤ 0.07; ○ linear estimate, % RH = 100.